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Skin Calcium-Binding Protein in Squamous Metaplasia of Human Uterine Cervix

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The distribution of skin calcium-binding protein in squamous cell metaplasia of human endocervix, in normal human skin, and in ovarian cancer was determined by the immunofluorescence technique. A rabbit antiserum specific to rat SCaBP was characterized by Ouchterlony immunodiffusion and by immunoprecipitation of ^{125}I-labeled SCaBP. The specificity of antibody labeling was demonstrated by using preimmune rabbit serum and SCaBP antiserum competitively absorbed with purified SCaBP. In normal human skin SCaBP was found exclusively in the basal layer cell cytoplasm. This protein was not detected in normal columnar epithelium of endocervix. Epithelial tissues in the zone of transition between the cylindrical epithelium of the endocervical mucosa and the stratified squamous epithelium of the exocervix were obtained from 14 patients with a wide variety of squamous cell metaplasia. In the early stage of metaplasia SCaBP was detected exclusively in the cytoplasm of reserve undifferentiated cells. In the terminal stage of metaplasia the SCaBP was present only in the basal cell layer. SCaBP was found in several layers of dysplastic tissue, and this distribution appeared to be related to the loss of normal maturation of the epithelium. SCaBP was also present in squamous cell carcinoma of endocervix especially in the least differentiated regions of the tumor. No SCaBP was detected in any ovarian cancer cells. These findings are potentially useful as a means of early detection of squamous metaplasia and of distinguishing premalignant anaplastic lesions from those that are benign and nonproliferative. In addition, the presence of SCaBP in tumors derived from metaplastic epithelia and its absence in the ovarian cancer indicate that immunohistochemical search for this protein might be of value in tumors in which an epidermoid origin is a possibility.
(Am J Pathol 1984, 114:454–460)

WE HAVE recently demonstrated that a vitamin-D-dependent calcium-binding protein is present in rat skin (SCaBP).[1-3] This protein is located in the cytosol of the basal layer cells, which are the proliferative cells of the epidermis.[4] SCaBP shares many of the properties of other intracellular calcium-binding proteins.[5] It is a low-molecular-weight protein (12,000 daltons), is acidic in nature, and has an amino acid composition very similar to that of other calcium-binding proteins. It has a high content of phenylalanine, and there are striking homologies in the amino acid sequence between this protein and parvalbumin.[3] Like parvalbumin, each molecule binds two calcium ions with high affinity.[3] There are, however, differences in the primary structure and calcium-binding properties between SCaBP and other low molecular weight calcium-binding proteins.[3] Hence, it is not surprising that antisera raised against SCaBP do not cross-react with intestinal CaBP, calmodulin, parvalbumin, or the S-100 protein.[1,2,4,6] The function of SCaBP is not known but it is distributed in the basal layer of all squamous epithelia thus far examined.[6] These epithelial tissues undergo cell division essentially in the basal layer; and when the basal layer cells rise to suprabasal layer, they lose this capability of division as well as SCaBP content, as judged by immunofluorescence techniques.[4,6] This association suggested that SCaBP might be related to and be a marker for immature squamous epithelial cells capable of proliferation.

In several pathologic disorders, columnar epitheli-

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um assumes the characteristics of squamous epithelium, a process termed "epidermization" or "squamous cell metaplasia." This raised the question of whether or not such a metaplastic transformation might be associated with the appearance *de novo* of the SCaBP previously demonstrated in the basal layer of native squamous epithelia.[6] In the present paper, we report evidence for the presence of SCaBP in the metaplastic lesions of columnar epithelium of the endocervix.

Materials and Methods

Normal human skin and carcinoma of ovary were obtained from surgical specimens. Epithelial tissues in the zone of the endocervical cleft were taken during colposcopic investigation. The biopsies were frozen in liquid nitrogen and mounted on the cryostat chuck. Four-micron sections were cut with a universal microtome for immunohistological incubation. Several adjacent sections were stained with hematoxylin and eosin (H&E). The presence of stratified or epidermoid epithelium in the tunnel or clefts of endocervix was considered as a state of epidermization or squamous cell metaplasia. Morphologic criteria were used to distinguish between various degrees of atypism in the case of dysplasia: the detection of "reserve" or undifferentiated basal type cells, uniform in type rather than stratified, was interpreted as an initial stage of metaplastic lesion or "reserve" cell hyperplasia. The presence of multilayered patterns with a morphologic characteristic of stratified squamous epithelia was considered the terminal stage of metaplasia. The multilayered pattern with the presence of variably atypical basal type cells in the upper layers was considered dysplasia. Total or partial cell replacement was taken as the criterion of the gravity of lesion. In cases of mild atypism the atypical cells extended about one-half of the thickness of the epithelial layer. In those of marked atypism these cells penetrated through 75–90% of the epithelial thickness. In 2 patients, the histologic pattern corresponded to an epidermoid variety of cervical cancer (squamous cell carcinoma). In 4 patients selected for this study, the tissue sections of endocervix containing normal columnar epithelium served as controls.

Antiserum

A rabbit antiserum containing antibodies to rat SCaBP was obtained by methods previously described.[1] Rat skin samples were frozen in liquid nitrogen, pulverized in a Spex Mill at −80 C (Spec Inc., Metuchen, NJ), extracted with Tris buffer (1.37×10^{-2} M Tris, 0.119 NaCl, 4.74×10^{-3} M KCl, pH 7.4) and chromatographed on a Sephadex G 75 column. The fractions were analyzed for protein[6] and for calcium-binding activity by the Chelex 100 competitive binding assay.[8] Further purification was obtained using DEAR Sephadex A 25 ion exchange and Sephacril G 200 chromatography. After purification, SCaBP produced one band on dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the region of 12,000 daltons.[2] An antiserum to the purified pro-

Table 1—Skin Calcium-Binding Protein Immunofluorescence in Native and Metaplastic Squamous Epithelia

Organs	Histology	Number of specimens examined	Number of SCaBP-positive specimens	Localization of SCaBP staining
Skin	Normal epidermis	50	50	Basal cell layer
Endocervix	Normal columnar epithelium	4	0	
	Undifferentiated "reserve" cell hyperplasia	2	2	Undifferentiated cell layer
	Terminal stage of metaplasia	4	4	Basal cell layer
	Dysplasia with mild atypism	5	5	Basal and suprabasal cell layer, parallel to penetration of immature cells well
	Dysplasia with marked atypism	1	1	In all cellular layers
	Squamous cell carcinoma	4	4	In tumor cells mainly in the periphery of the tumoral buds
Ovary	Undifferentiated small-cell carcinoma	1	0	

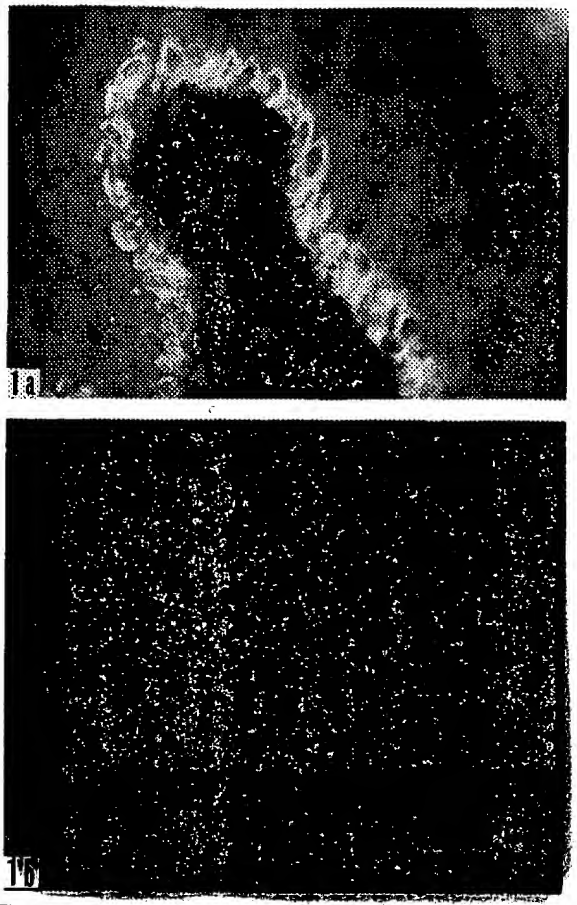


Figure 1 – Human skin. a – Epidermal basal cell layer of epidermis stained with SCaBP antiserum (diluted 1:40). (×625) (With a photographic reduction of 38%) b – Control section treated with preimmune rabbit serum (serum diluted 1:40) (×250) (With a photographic reduction of 31%)

tein was raised by repeated injection of white New Zealand rabbits with 50–100 μg of SCaBP. The antiserum had been previously tested by the Ouchterlony double immunodiffusion technique. The antibody precipitation technique was also used to determine the specificity of the antiserum, as previously described.[6] Cross-reactivity between rat and human SCaBP was tested by radioimmunoassay measurement of SCaBP in man by the use of rat antibody (unpublished) and by the immunoblotting technique, in which the rat antibody revealed the identical single band for SCaBP from rat and human skin (Siegenthaler, et al, submitted for publication).

For the control absorption, the antiserum against SCaBP was incubated with excess purified SCaBP or the total soluble protein extract of skin for 1 hour at 37 C and 12 hours at 4 C. After centrifugation at 2700*g* for 1 hour the supernatant was taken and used as a reagent for the indirect immunofluorescence procedure.

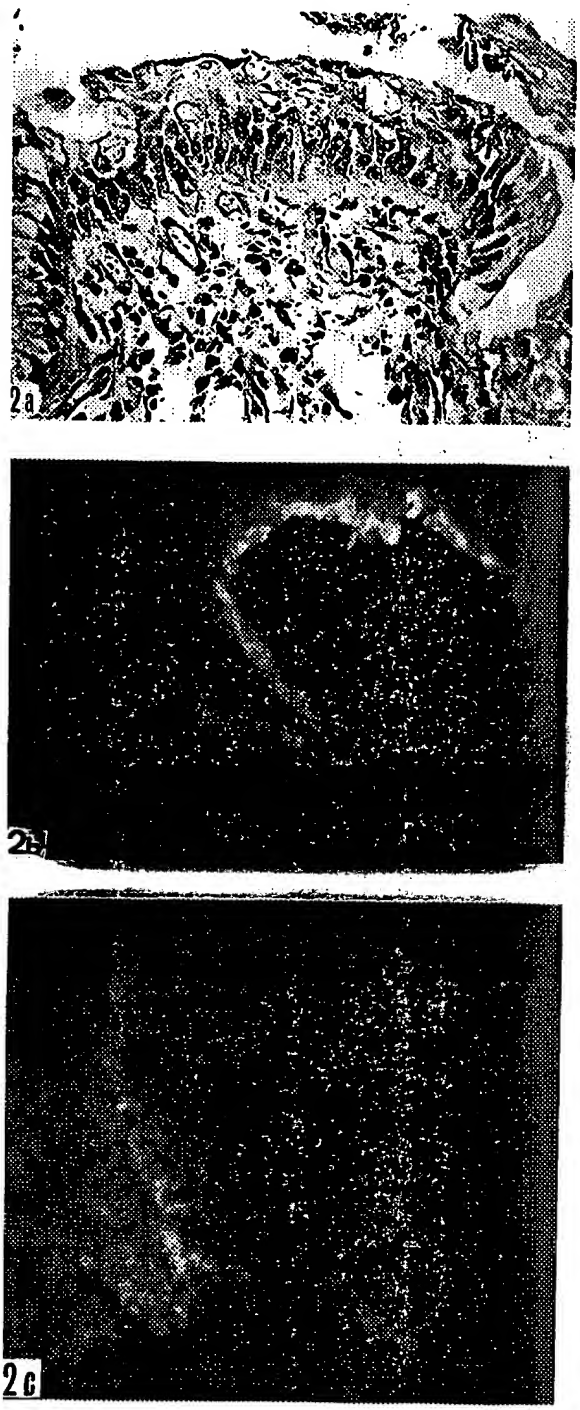
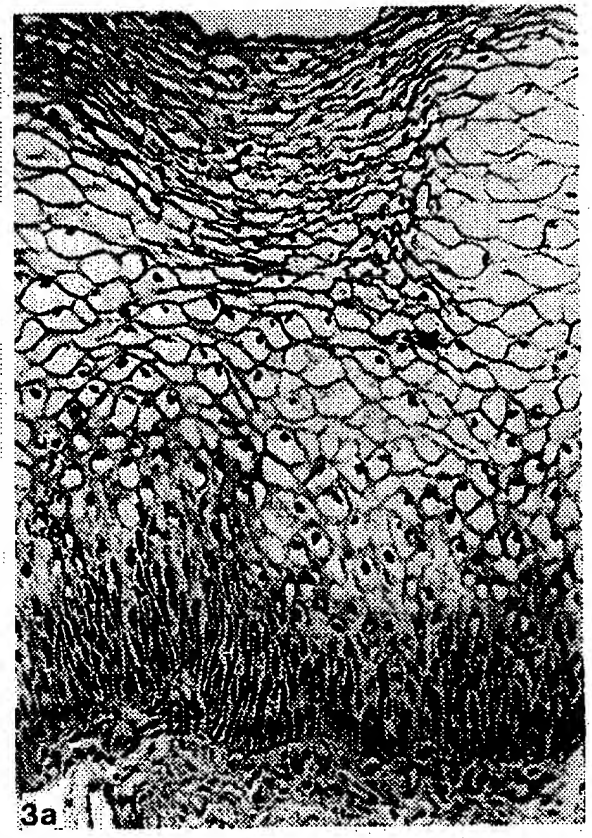


Figure 2 – Human uterine cervix. Hyperplasia of undifferentiated reserve cells. a – H&E staining. The hyperplasic "reserve" cells of basal type (*R*) forcing columnar epithelium upward toward the luminal area of cervical canal. (×300) (With a photographic reduction of 37%) b – Section of the same region. Immunofluorescence staining due to anti-SCaBP serum. (×625) Specific fluorescence is seen exclusively in the hyperplastic reserve cell population (*arrow*). No specific fluorescence is observed in the columnar epithelium of endocervix. (With a photographic reduction of 44%) c – Control section treated with preimmune rabbit serum. (×625) No fluorescence in the proliferative reserve cells is observed. (With a photographic reduction of 18%)



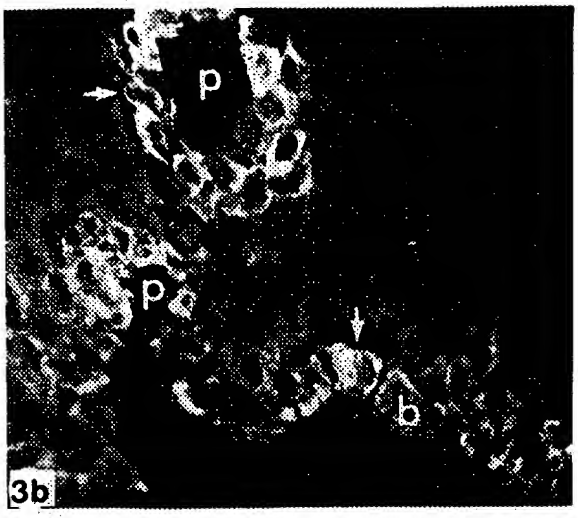


Figure 3—Human uterine cervix. Terminal stage of metaplastic transformation. **a**—H&E-stained section (×300) showing the morphologic details of squamous epithelium. Basal layer cell; *DP*, papilla. (With a photographic reduction of 23%) **b**—Section of the same region treated with SCaBP antiserum. (×625). Immunofluorescent staining is seen in the basal layer cells (*b*) of newly formed squamous epithelia of metaplasia; fluorescence in the basal layer cells of papilla (*P*). (With a photographic reduction of 20%) **c**—Control section treated with preimmune rabbit serum (×625). Notice the absence of fluorescence in the basal layer. (With a photographic reduction of 20%)

Ouchterlony double immunodiffusion was used for the testing of cross-reactivity. The antigens used were 1) brain S-100 calcium-binding protein (gift of Dr. Labourdette, Laboratoire de Neurochimie, Strasbourg, France) and 2) ram testis calmodulin (gift of Dr. Demaille, Centre de Recherche de Biochimie Macromoléculaire, Montpellier, France). The purified rat intestinal SCaBP was prepared as described by March et al.[9]

Indirect Immunofluorescence

The presence of SCaBP in endocervix was determined only by indirect immunofluorescence,[4] because insufficient biopsy tissue was obtained for the immunoprecipitation technique or radioimmunoassay. For the indirect immunofluorescent test, the tissue sections were incubated with the SCaBP antiserum (diluted 1:10 to 1:640 in phosphate-buffered saline (PBS) for 30 minutes at room temperature). After washing for 30 minutes in PBS, tissues were incubated for 30 minutes with commercially prepared fluorescein isothiocyanate (FITC)-labeled sheep anti-rabbit IgG (H&L) antiserum (Hoescht-Boehring, Paris, France): specific antibody concentration, 1 mg/ml; FITC protein ratio, 2.2; working dilution, 1:20 in PBS to obtain 50 μl/ml of specific antibodies. The slides were then washed for 30 minutes in PBS and mounted with glycerol. They were examined with a Leitz Orthoplan microscope equipped for incident illumination. Con-

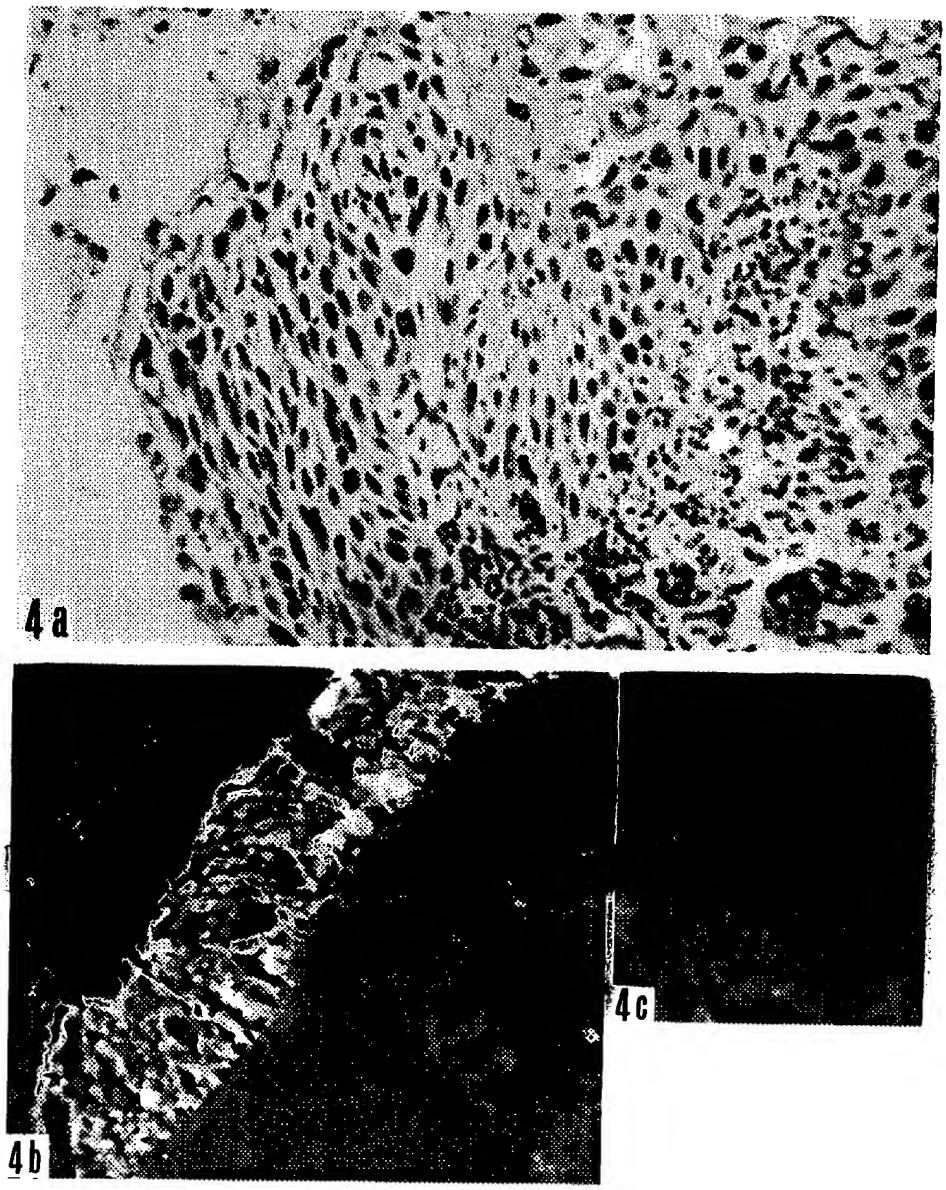


Figure 4—Human uterine cervix. Dysplasia with marked atypism (full thickness lesion). a—H&E-stained section showing uniformly small immature cells with a high nuclear/cytoplasmic ratio (×300). (With a photographic reduction of 5%) b—Section of a similar region treated with SCaBP antiserum (×250) Notice the immunospecific fluorescent staining in all cellular layers (*arrow*). (With a photographic reduction of 17%) c—Control section treated with preimmune rabbit serum (×250) No fluorescence is observed. (With a photographic reduction of 17%)

trol sections were stained with preimmune serum from the corresponding rabbit or with antiserum against SCaBP absorbed with the antigen as described above.

In order to avoid the artificial migration of water-soluble antigen, the freeze-drying method of fixation was used for control as previously described.[4]

Results

SDS-PAGE of immunoprecipitated ^{125}I counts co-migrate with the ^{125}I-SCaBP marker, showing a single band of radioactivity corresponding to a molecular weight of 12,000. In Ouchterlony double immunodiffusion the antiserum against SCaBP recognized the antigen from protein extract of homogenized skin. No precipitation lines were observed when SCaBP antiserum was incubated with protein extract of muscle, intestine, liver, bone, or kidney. SCaBP antiserum did not cross-react with ram testis calmodulin, brain S-100, or intestinal CaBP (not shown).[4,6]

The location of SCaBP of native and metaplastic squamous epithelium is shown in Table 1. In the normal human skin anti-SCaBP serum gave bright staining of the epidermal basal cell layer (Figure 1). In agreement with our earlier observation in the rat,[6] the pattern of basal cell immunoreactivity in human skin was consistent with a cytoplasmic staining. The dis-

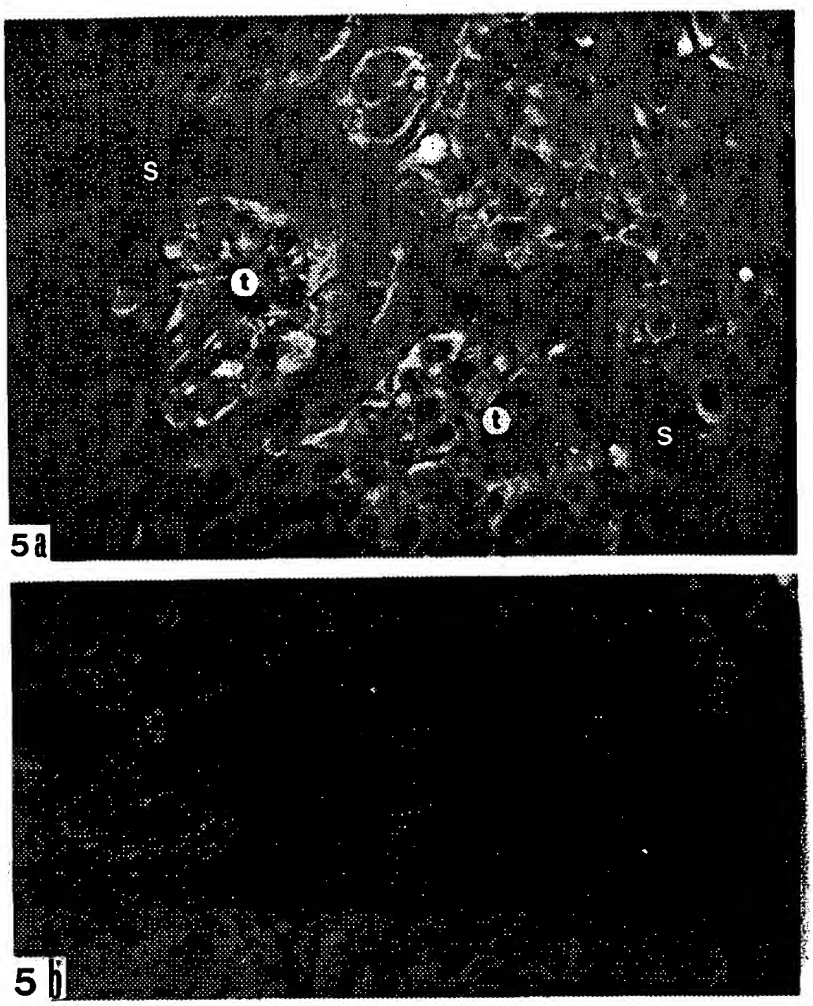


Figure 5—Human uterine cervix. Squamous cell carcinoma. a—Section treated with SCaBP antiserum. (×625) Notice the immunofluorescence in tumor cells mainly in the periphery of the tumor buds. *s*, stroma; *t*, tumor bud. b—Control section treated with preimmune rabbit serum. (×250) No fluorescence is observed.

tribution of the antigen in the tissue prepared by freeze-drying was similar to that obtained using the nondried freezing technique. The same distribution, but with lower intensity of specific immunofluorescence, was seen in methanol-fixed sections. No specific SCaBP antibody immunofluorescent staining occurred with SCaBP antiserum that had been competitively absorbed with purified protein or with total protein extract from rat skin.

The normal endocervical tissue did not contain SCaBP. In all cases, zones of squamous metaplasia contained SCaBP antigen. The cytologic distribution of SCaBP, as detected immunohistochemically, was variable. In the case of the initial state of squamous metaplasia, the SCaBP was detected only in the cytoplasm of the hyperplastic undifferentiated cells (Figure 2). In the terminal stage of metaplasia, the specific fluorescence was seen exclusively in the cytoplasm of the basal layer of the metaplastic region (Figure 3).

On the surface of all dysplastic lesions, the SCaBP immunoreactivity was detected in the small uniform, immature cells. The distribution of SCaBP was parallel to the penetration of this immature type of cell well into the upper layer of epithelium. In the samples considered as having dysplasia with mild atypism, the SCaBP was demonstrated only in the lower layer of immature cells. No staining was observed in the upper layers. In the state of dysplasia with marked atypism ("full thickness" alteration), SCaBP was demonstrated in all cellular layers (Figure 4).

In squamous cell carcinoma SCaBP immunofluorescence was present exclusively in the tumor cell population (Figure 5). No immunoreactivity was observed in the stroma. The percentage of stained cells varied in the different areas of the lesion. The majority of the SCaBP-positive cells was present in the periphery of the tumoral buds in the zones that may be considered histologically as least differentiated. However, several SCaBP-positive cell agglomerations were present in the areas that were histologically more

differentiated. No SCaBP immunoreactivity was present in any region of undifferentiated ovarian cancer.

Discussion

SCaBP, a calcium-binding protein originally isolated from rat skin is distributed exclusively in the undifferentiated basal cells of squamous epithelia.[6] The present study made in human tissue confirms the earlier observations in rats that SCaBP is present in normal squamous but not in normal columnar epithelia. Moreover, in the case where columnar epithelium assumes the characteristics of squamous epithelium, the *de novo* appearance of SCaBP takes place. This protein may be, therefore, regarded as a common marker of squamous transformation. This finding may have immediate relevance to the detection of the early lesions of squamous metaplasia, especially those in which the morphologic picture is confusing.

The cytologic distribution of SCaBP in the variety of metaplastic lesions of endocervix suggests that its location depends on the degree of squamous epithelial cell maturity. In the terminal state of metaplasia the SCaBP is present only in the basal layer cells, but the multilayered pattern is found in the state of dysplasia, related to loss of normal maturation of the epithelium. This finding may be useful as a means for differentiating premalignant anaplastic lesions from those that are benign and nonproliferative. In addition, the presence of SCaBP in tumors derived from metaplastic epithelia and its absence in ovarian cancer indicate that the immunohistochemical search for this protein might be of value in tumors in which an epidermoid origin is in question.

It is possible that the presence of SCaBP in basal cells of either native or metaplastic stratified epithelia reflects a specific aspect of its function in the maintenance of the undifferentiated proliferative cells in the basal layer of squamous epithelium. It is interesting to note that epidermal and metaplastic cervical epithelia have an unusual response to changes in extracellular calcium concentrations.[10,11] In contrast to all other cell types examined, which cease to replicate when extracellular calcium levels are decreased below 0.1 M,[12] these cells proliferate only at low calcium concentrations. Higher calcium levels in the culture medium inhibit their mitotic activity and induce stratification and squamous differentiation. It is tempting to speculate that there is a causal relationship between the appearance of SCaBP and the cellular response to calcium concentrations. If this were so, then the metaplastic transformation of cervical epithelium may involve altered cellular calcium metabolism.

Finally, several lines of evidence indicate that many neoplastic cells *in vitro* do not respond by modification in mitotic rate to changes in extracellular calcium concentrations, in contrast to normal cells.[12,13] It has also been shown that the regulation of growth and differentiation by calcium is altered in malignant epidermal cells and preneoplastic cells exposed to carcinogenesis.[14] If the SCaBP expression of SCaBP is altered in aberrant differentiation states of tumor cells, as suggested by our results, this protein could be linked to decreased dependence of neoplastic cells on extracellular calcium for growth regulation.

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